

Stable Accumulation of *Aspergillus niger* Phytase in Transgenic Tobacco Leaves¹

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Phytase from *Aspergillus niger* increases the availability of phosphorus from feed for monogastric animals by releasing phosphate from the substrate phytic acid. A phytase cDNA was constitutively expressed in transgenic tobacco (*Nicotiana tabacum*) plants. Secretion of the protein to the extracellular fluid was established by use of the signal sequence from the tobacco pathogen-related protein S. The specific phytase activity in isolated extracellular fluid was found to be approximately 90-fold higher than in total leaf extract, showing that the enzyme was secreted. This was confirmed by use of immunolocalization. Despite differences in glycosylation, specific activities of tobacco and *Aspergillus* phytase were identical. Phytase was found to be biologically active and to accumulate in leaves up to 14.4% of total soluble protein during plant maturation. Comparison of phytase accumulation and relative mRNA levels showed that phytase stably accumulated in transgenic leaves during plant growth.

A key parameter in the development of a commercially viable process for the production of a recombinant protein is the ultimate yield of a recombinant protein from the host organism. There are many examples of the expression of valuable mammalian proteins and peptides in plants such as monoclonal antibodies (Hiatt et al., 1989; Düring et al., 1990), human epidermal growth factor (Higo et al., 1993), enkephalins (Vandekerckhove et al., 1989), interferon (DeZoeten et al., 1989), and HSA (Sijmons et al., 1991) or bacterial and fungal proteins such as α -amylase (Pen et al., 1992), T4 lysozyme (Düring et al., 1993), and phytase (Pen et al., 1993). So far, expression levels reported for heterologous (poly)peptides in transgenic plants range from 0.001% of total soluble protein for human epidermal growth factor (Düring et al., 1990) to 2.9% of total soluble protein for enkephalins (Vandekerckhove et al., 1989).

The factors that influence the yield of a particular heterologous protein are complex and include transcriptional factors such as promoter activity and site of integration of the T-DNA in the chromosome (Dean et al., 1988). But it has become increasingly clear that posttranscriptional fac-

tors (Kuhlmeier, 1992; Sullivan and Green, 1993) also play an important role in the control of gene expression. An organism can regulate gene expression at multiple levels, including mRNA splicing, 3'-end formation, stability, and translation as well as posttranslational factors, such as protein stability (De Clercq et al., 1990) and protein modification (Kornfeld and Kornfeld, 1985). In terms of cost effectiveness for producing biomass, the growing of crops in the field, in general, can compete with any other system: it is inexpensive, it can be done in bulk quantities, and it requires limited infrastructure. Enzymes, e.g. those in food, feed, or processing industries, are products that can be produced economically in genetically engineered plants.

Here we report the high-level accumulation of biologically active phytase from *Aspergillus niger* in transgenic tobacco plants. Phytases (*myo*-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8) are enzymes that catalyze the hydrolysis of phytic acid. Phytic acid (*myo*-inositol hexaphosphoric acid) is the main storage form of phosphorus in many seeds. Up to 80% of the total phosphorus in seeds has been reported to be in the form of phytate (Lolas and Markakis, 1977). When used as feed, phytate phosphorus passes through the gastrointestinal tract of monogastric animals and is excreted in the manure. As a consequence, a significant amount of nutritionally important phosphorus is not utilized by monogastric animals. The excreted phosphorus contributes to the phosphate pollution problem in areas with intensive livestock farming. Addition of phytase to feed has been shown to optimize phosphorus utilization and to reduce excretion of phosphorus in the manure of pigs and poultry (Simons et al., 1990).

MATERIALS AND METHODS

Plasmid Construction and Plant Transformation

Binary vector pMOG413 (Pen et al., 1993) contains a chimeric phytase gene encoding the tobacco PR-S signal peptide (Cornelissen et al., 1986) and a cDNA fragment encoding the mature *Aspergillus niger* phytase gene (Van

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Abbreviations: EF, extracellular fluid; EndoH, endo- β -N-acetyl-glycosaminidase H; HSA, human serum albumin; PR-S, pathogen-related protein S; SSU, small subunit of Rubisco; TFMS, trifluoromethanesulfonic acid.

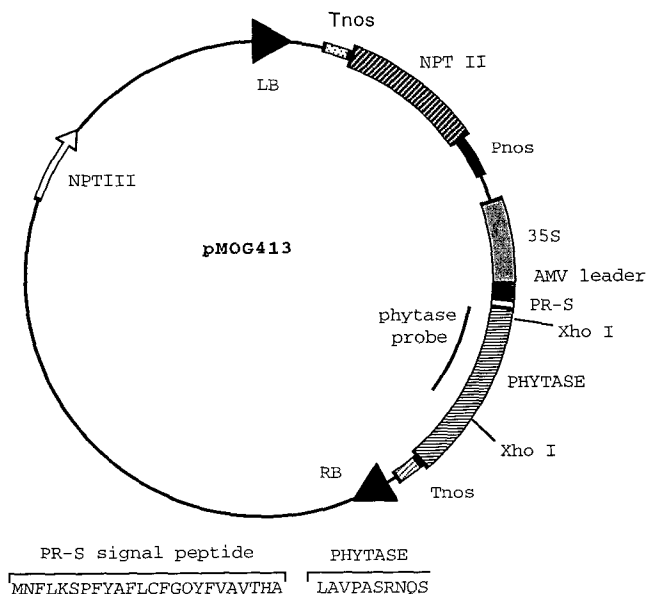


Figure 1. Schematic representation of binary vector pMOG413, containing bacterial neomycin phosphotransferase III gene (NPTIII), left border sequence (LB), nopaline synthase terminator (Tnos), neomycin phosphotransferase II gene (NPTII), nopaline synthase promoter (Pnos), cauliflower mosaic virus 35S promoter with double enhancer sequence (35S), RNA4 leader sequence of alfalfa mosaic virus (AMV leader), signal peptide from tobacco PR-S protein (PR-S), phytase coding region (PHYTASE), nopaline synthase terminator (Tnos), and right border sequence (RB). Indicated by an arc is the 1-kb *Xho*I fragment used as a probe in northern hybridization. Below the plasmid, the amino acid sequence of the exact fusion between PR-S and phytase is shown.

Hartingsveldt et al., 1993) (Fig. 1). It has been shown that the tobacco PR-S signal peptide functions in protein secretion in plants when attached to heterologous proteins and that it is cleaved correctly upon secretion (Sijmons et al., 1991). The chimeric gene was placed under the control of a modified cauliflower mosaic virus 35S promoter fragment (Guiley et al., 1982) with a duplicated enhancer sequence (nucleotides -343 to -90 relative to the transcription start site). Duplication of the enhancer has been shown to increase promoter activity (Kay et al., 1987).

Downstream of the promoter fragment, a synthetic leader sequence derived from alfalfa mosaic virus RNA4 was inserted. It has been reported that mRNA translation can be enhanced by this viral leader sequence (Brederode et al., 1980; Jobling and Gehrke, 1987). The phytase gene was terminated by the termination sequences of the nopaline synthase gene from *Agrobacterium tumefaciens*. This binary vector was mobilized into *A. tumefaciens* LBA 4404 in a triparental mating mediated by *Escherichia coli* strain HB101 containing plasmid pRK2013 (Ditta et al., 1980). Tobacco (*Nicotiana tabacum* cv Petit Havana SR1) leaf discs were transformed with pMOG413 according to the method described by Horsch et al. (1985) using the binary vector system (Hoekema et al., 1983). Transgenic plants were selected on medium containing 100 mg/L kanamycin.

Phytase Assay

Leaf samples were taken from primary transformants 3 weeks after transfer to the soil, or from S_1 plants each week during 7 weeks of plant maturation. Leaf tissue was homogenized in 25 mM sodium acetate buffer, pH 5.5, containing 0.1 mM CaCl_2 . The homogenate was centrifuged for 5 min in an Eppendorf centrifuge, and the supernatant was collected. The soluble protein content of this crude extract was determined (Bradford, 1976). Phytase activity was determined in a sample containing 5 μg of protein (Simons et al., 1990) and compared with a standard curve of *A. niger* phytase mixed with an equal amount of protein from non-transgenic leaves to determine the amount of phytase present. One unit of phytase is the activity that liberates 1 μmol of phosphate from phytic acid in 1 min. To determine the phytase level per mg dry weight, fresh leaf samples were cut in half. One part was lyophilized to determine the dry weight, and the other part was used to determine the phytase level per mg fresh weight.

Western Blotting

Polyclonal antibodies were raised in a rabbit against denatured phytase from *A. niger*. Leaf protein samples were electrophoresed on 10% polyacrylamide-SDS gels and subsequently blotted in a semidry blot apparatus onto nitrocellulose in 25 mM Tris, 192 mM Gly, pH 8.3. Blots were blocked by immersing the membrane in 5% dried milk in PBS containing 0.5% Tween 20 for 1 h at room temperature, subsequently incubated with phytase polyclonal antiserum for 1 h, and finally incubated with rabbit anti-goat peroxidase conjugate for 1 h. Blots were developed using the ECL detection system as described by the manufacturer (Amersham).

Deglycosylation of Protein Extracts

EndoH of *Streptomyces plicatus* from recombinant *E. coli* was used for the deglycosylation of protein extracts. Protein extracts were incubated twice for 3 h at 37°C with 2 milliunits of EndoH in a 0.05 M sodium acetate buffer, pH 5.5, containing 0.2% SDS, 10 μM pepstatin A, and 1 mM PMSF. After digestion, the samples were dried in a Speed-Vac SC 1000 (Savant Instruments, Farmingdale, NY). Chemical deglycosylation was done with TFMS (Edge et al., 1981), and then the samples were finally lyophilized. To exclude inhibition of EndoH by plant substances, *A. niger* phytase was mixed with tobacco leaf extracts in identical ratios and used as a control in EndoH treatment.

Isolation of EF

Lower leaves of transgenic plants grown in soil for 4 weeks were cut in longitudinal sections of about 1 cm, rolled into Parafilm (American National Can, Greenwich, CT), mounted in a 10-mL syringe, and vacuum-infiltrated twice for 10 min in 5 mM Hepes, pH 6.3, containing 50 mM NaCl. Adhering fluid was removed at 200g, and the extracellular fluid was collected by centrifugation at 600g for 10 min. The fluid was assayed for marker peroxidase activity

(Hendriks et al., 1985), phytase activity, and protein content (Bradford, 1976).

RNA Analysis

Total RNA was isolated from transgenic S_1 plants (Verwoerd et al., 1989) that were germinated on kanamycin-containing medium and grown in soil for 1, 2, or 4 weeks. Phytase transcripts were analyzed by northern blotting, using 10 μ g of total RNA per lane. For detection of phytase transcript, a random-primer-labeled 1-kb *Xho*I internal phytase fragment was used as a probe, and for the controls, a SSU probe from tobacco (Mazur and Chui, 1985) and an actin probe from rice (McElroy et al., 1990) were used. T7 run-off transcripts of the PR-S phytase gene were made in vitro as follows (Krieg and Melton, 1984). The entire PR-S phytase coding sequence and part of the alfalfa mosaic virus leader sequence, slightly modified for cloning purposes, were cloned in the T7 vector pBluescript (Stratagene) as an *Avr*II-*Pst*I fragment. The T7 transcripts made in vitro were used as standards to quantify the phytase mRNA levels.

Immunocytochemistry

Leaf samples of transgenic and nontransgenic tobacco plants were fixed by infiltration of 2% (w/v) glutaraldehyde:3% (w/v) paraformaldehyde in phosphate-citrate buffer (0.1 M $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$, 9.7 mM citric acid $\cdot \text{H}_2\text{O}$, pH 7.2, 1.5 mM CaCl_2) for 2 h at room temperature under vacuum. The samples were then washed, dehydrated in ethanol, and embedded in LR Gold resin (London Resin Co., London, UK) under low temperature as described by Van Lent et al. (1990). Thin sections were immunogold labeled with polyclonal antibodies to phytase and preimmune serum, in a dilution of 1:1000, and protein A-gold complexes with 10-nm-diameter gold particles (Van Lent et al., 1990). Specimens were observed and photographed with a Philips (Eindhoven, The Netherlands) CM12 transmission electron microscope.

RESULTS AND DISCUSSION

Phytase Expression Analysis

A total of 72 independent kanamycin-resistant shoots obtained from transformation experiments with the phytase expression vector pMOG413 were transferred to the soil. Transformed plants were grown for 3 weeks in the greenhouse, and leaf samples were tested for phytase activity. The highest expression level obtained was 1.7% of total soluble protein in leaves, of plant line MOG413.55. This plant line was used in all further described analyses and is referred to as line A. The average expression level in all 72 plants was 0.2%. Western blotting (Fig. 2) with polyclonal antibodies raised against the *A. niger* enzyme demonstrated that the observed phytase activity was caused by expression of the heterologous enzyme in leaves.

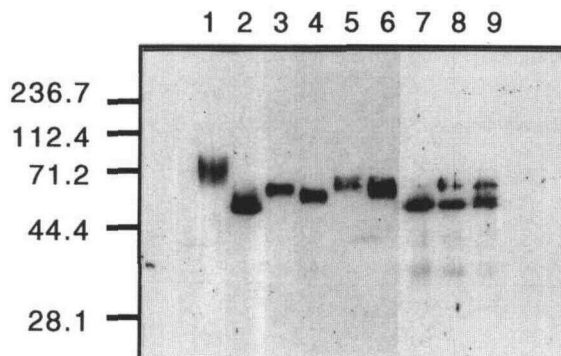


Figure 2. Deglycosylation of phytase produced in tobacco seeds and leaves with EndoH and TFMS. The amount of protein loaded onto the gel was determined before treatment. Lanes 1, 2, and 7, 30, 60, and 75 ng of *A. niger* phytase; lanes 3, 4, and 8, 1, 2, and 2.5 μ g of crude seed extract from plant line MOG413.25; and lanes 5, 6, and 9, 1, 2, and 2.5 μ g of crude leaf extract from plant line MOG413.25. The treatments were: lanes 1, 3, and 5, nontreated; lanes 2, 4, and 6, treated with EndoH; and lanes 7, 8, and 9, treated with TFMS. Molecular masses ($\times 10^{-3}$) of prestained marker proteins (BRL) are indicated on the left. MOG413.25 had an expression level comparable to that of MOG413.55.

Deglycosylation

The molecular mass of tobacco phytase from leaves was approximately 70 kD, compared to approximately 80 kD for the *Aspergillus* enzyme (Fig. 2). The difference in molecular mass was found to be caused by differences in glycosylation. A total of 10 potential Asn-linked sites are present in the primary structure, and the *A. niger* enzyme is known to be heavily glycosylated (Van Hartingsveld et al., 1993). Crude protein extracts from leaves and seeds were treated with EndoH or deglycosylated with TFMS and subsequently subjected to western blotting. Deglycosylation with EndoH resulted in a decrease of the molecular mass of phytase from leaf to approximately 67 kD. The molecular mass of phytase from seeds was approximately 68 kD and decreased to approximately 64 kD after EndoH treatment. Treatment of phytase from seeds and leaves with TFMS resulted in a protein with an apparent molecular mass of approximately 60 kD on SDS-polyacrylamide gels, identical to that of the deglycosylated *Aspergillus* enzyme (Fig. 2). Comparison of the deglycosylation with EndoH and TFMS showed that both leaf- and seed-derived phytase contained high-Man and complex-type carbohydrate chains. Tissue-dependent differential glycosylation of proteins, as found here for seeds and leaves, has been described before (Kornfeld and Kornfeld, 1985). Despite the differences in glycosylation, the specific activity of the purified leaf-derived phytase was found to be identical to that of the purified *A. niger* enzyme (data not shown).

Extracellular Targeting

Extracellular fluid was isolated from leaves of S_1 plants of the highest-expressing line (line A) to determine whether the PR-S signal peptide functioned in secretion of phytase. The specific phytase activity (as expressed in

Table 1. Extracellular targeting of phytase

Plant Line	Phytase	Peroxidase
	EF/total ^a	EF/total ^b
SR1	1.0	96.1
A.5	87.0	72.9
A.7	52.6	57.9
A.9	55.4	71.2

^a Ratio of phytase activity measured in EF and total leaf extract.

^b Ratio of peroxidase activity measured in EF and total leaf extract.

units/mg total soluble plant protein) in the extracellular fluid was found to be approximately 87-fold higher than in total leaf extract (Table 1), showing that the enzyme was secreted. The ratio of specific activity in extracellular and total extract was similar for phytase and peroxidase (Table 1), a marker enzyme for the extracellular fluid, and this is further evidence for secretion of the enzyme.

Immunocytochemistry

Plants of transgenic line A were used for localization of phytase by immunocytochemistry. Figure 3 illustrates that phytase is exported to the extracellular space. Controls consisted of the phytase-expressing tobacco line incubated with preimmune serum, nontransgenic SR1 plants incubated with polyclonal antibodies raised against *A. niger* phytase, and nontransgenic SR1 plants incubated with pre-immune serum. All controls were negative.

RNA Analysis

Three S₁ plants of line A were germinated on kanamycin-containing medium and grown in soil for 1, 2, or 4

weeks, and total RNA and DNA were isolated. Southern analysis of primary transformant line A showed the presence of four phytase copies. S₁ plant line A.7 also had four copies, and plant lines A.5 and A.9 both had three copies but were not identical (data not shown), explaining the difference in phytase expression levels and northern blot signals between the tested plants. A northern blot was hybridized with a 1-kb *Xho*I internal phytase fragment (Fig. 4A). During plant maturation, the hybridization signal of phytase mRNA increased. As a control, the same blot was hybridized with probes for SSU and actin probes from tobacco. Both genes are highly expressed in green tissue (Fig. 4, B and C). For both actin and SSU, the level of mRNA decreased during plant maturation, which may be explained by a decrease in metabolic activity of aging plants. Because the total amount of RNA declines during growth, it is likely that the absolute amount of phytase mRNA is not increasing, as suggested by the results of northern blots. To allow estimates of the absolute amount of phytase mRNA, the hybridization signal of phytase mRNA in transgenic plants was compared with T7-RNA run-off transcripts. For the S₁ plant line A.5 grown for 4 weeks in soil, which showed the strongest hybridization signal, the mRNA level was approximately 0.02% of total mRNA. This level is comparable to the maximum mRNA expression level found in transgenic plants expressing HSA (Sijmons et al., 1991). These plants produced a maximum level of approximately 0.02% HSA per mg total soluble protein. Data from HSA-expressing plants are derived from previous experiments, but plant growth conditions and sample handling are comparable to those of phytase-expressing plants.

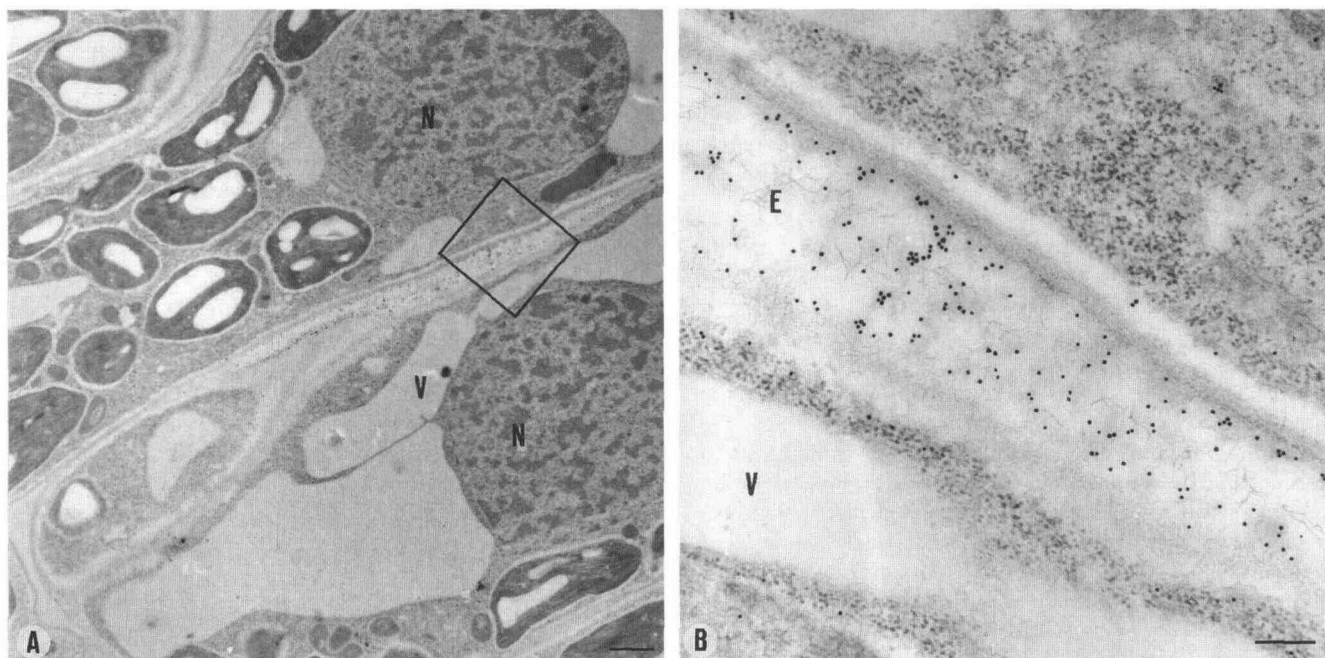


Figure 3. Electron micrographs showing the immunogold localization of phytase exclusively in the extracellular space of transgenic tobacco leaf tissue. V, Vacuole; N, nucleus; E, extracellular space. B, Detail of the boxed area in A. Bars represent 1 μ m (A) and 200 nm (B).

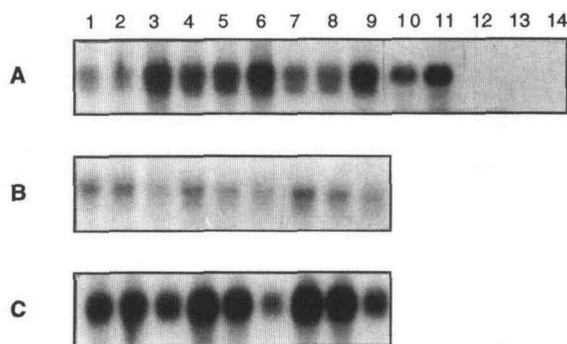


Figure 4. Northern hybridization of 10 µg of total RNA from phytase transgenic plants. Lanes 1, 4, 7, and 12, RNA from 1-week-old plants from lines A.5, A.7, A.9, and SR1 control. Lanes 2, 5, 8, and 13, RNA from 2-week-old plants from lines A.5, A.7, A.9, and SR1 control. Lanes 3, 6, 9, and 14, RNA from 4-week-old plants from lines A.5, A.7, A.9, and SR1 control. Lanes 10 and 11, Fifty and 100 ng of T7 phytase run-off transcripts. A, Lanes hybridized with a phytase probe; B, lanes hybridized with an actin probe; C, lanes hybridized with an SSU probe.

Phytase Accumulation

The three S_1 transgenic plants of line A (A.5, A.7, and A.9) were used to follow phytase accumulation during growth of the plants. Leaf samples were collected each week during growth of the plants in the greenhouse from 1 until 7 weeks after transfer to soil. Samples taken at weeks 1, 2, and 4 were used to compare phytase mRNA and protein levels. It was impossible to isolate intact RNA from 5-week-old plants, due to senescence of the tobacco leaves. Leaf samples taken later than 5 weeks thus were used only for phytase activity assays. The results show (Table II) that the amount of total soluble protein per dry weight decreased approximately 6-fold in 3 weeks. The phytase levels, based on dry weight, increased during the first 5 weeks and remained stable during the following 2 weeks. Because it can be expected that after 5 weeks, due to senescence of the tobacco leaves, the phytase mRNA level will decrease, phytase must be resistant to proteolytic enzymes in this period. From these results it can be concluded that phytase stably accumulated during plant maturation. Based on the amount of enzyme per soluble protein, the phytase level increased approximately 20-fold in a 7-week

period, up to a maximum level of 14.4%. This also indicates that phytase is extremely stable. Although HSA-expressing plants and phytase-expressing plants showed a comparable expression on the RNA level, phytase-expressing plants produced 1.7% phytase per mg total soluble protein, whereas HSA-expressing plants produced approximately 0.02% HSA per mg total soluble protein at approximately the same time during growth. This 85-fold difference in protein:mRNA ratio between phytase- and HSA-expressing plants indicates either that translation of the phytase mRNA in tobacco is very efficient compared to HSA or that phytase is more stable than HSA at the protein level.

Translation is regulated primarily by translation initiation, translation termination, and codon usage pattern of the coding region. For both the HSA and phytase construct, the same fusion of the alfalfa mosaic virus RNA4 leader sequence to the PR-S signal sequence was used, and at the 3' end the same nopaline synthase terminator was used, suggesting that translation initiation and translation termination are comparable for both constructs. Although there is some difference in codon usage patterns between HSA and phytase, neither is significantly different from that used by dicotyledonous plant genes in general (Campbell and Gowi, 1990), indicating that there is no proof of more efficient translation of the phytase mRNA compared to the HSA mRNA. Phytase is known to be heavily glycosylated (van Hartingsvedt et al., 1993). This can explain why the protein is much more stable than, for example, HSA, which shows a comparable mRNA level. From the literature it is known that carbohydrate chains can play a role in the stability of some glycoproteins because they promote folding in the most stable three-dimensional structure (Faye et al., 1989).

The high yield of a recombinant protein from the host organism is essential for the development of a commercially viable process. The expression level of 14.4% phytase per mg total soluble plant protein is, to our knowledge, the highest level of a nonplant protein in transgenic plants ever reported. The plants containing phytase may be used in industrial processes requiring the action of phytase, such as the manufacturing of a feed additive for nonruminants (Pen et al., 1993). The phytase-producing plants can be used directly, without further processing, or the phytase

Table II. Phytase accumulation in transgenic tobacco plants

The results shown are the results of two independent assays (variation between the two assays was about 10%).

Plant Age	Percent Protein/ Dry Wt ^a	Line A.5		Line A.7		Line A.9	
		µg phytase/ mg protein	ng phytase/ mg dry wt	µg phytase/ mg protein	ng phytase/ mg dry wt	µg phytase/ mg protein	ng phytase/ mg dry wt
1 Week	12.50	16	1600	6	600	8	1200
2 Weeks	4.50	28	1200	20	900	30	900
3 Weeks	2.65	52	1700	74	1800	66	1800
4 Weeks	2.15	68	2000	142	2600	110	1700
5 Weeks	2.50	88	2500	122	3000	124	2200
6 Weeks	1.85	122	2500	116	2200	128	2200
7 Weeks	2.15	144	2400	126	2600	88	1900

^a Percentage protein per dry weight, average of three plants.

may be extracted and purified from the plants before application. Purification of phytase can best be done from 5-week-old plants, since at that time the absolute amount of phytase per mg dry weight is the highest. Older plants will accumulate high levels of terpenes and phenolic compounds, which might interfere with the purification procedure.

CONCLUSIONS

Recently, we demonstrated that phytase-containing tobacco seeds can be effectively used as a substitute for Pi in animal feed, showing that phytase produced in transgenic plants can be used as a feed additive for improvement of phosphorus utilization (Pen et al., 1993).

In this study, we showed that a chimeric phytase gene gave rise to transgenic tobacco plants expressing high levels of phytase. The constitutive expression of the enzyme did not obviously affect plant phenotype, since transgenics were indistinguishable from control plants, but this must be confirmed by more extensive analyses of plants grown in the field. Biologically active phytase was located extracellularly, as shown by immunocytochemistry and determination of the specific phytase activity in the extracellular fluid. Phytase mRNA and protein levels in transgenic plants were compared with the levels found in HSA-expressing plants (Sijmons et al., 1991). We conclude that translation of phytase mRNA is either very efficient compared to HSA mRNA, or phytase is more stable than HSA. We showed that phytase stably accumulated in maturing leaves, up to 14.4% of total soluble protein or 0.3% of dry weight. Even in senescent leaves, where most of the proteins are degraded, phytase is still present at a high level.

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